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Liquid chromatography with fluorimetric detection of triorganotin compounds in marine biological materials

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Abstract

Several extraction methods have been evaluated for marine biological materials with regard to tributyltin and triphenyltin determination by liquid chromatography with post-column fluorimetric detection using fisetin as a fluorogenic reagent. Extraction with ethyl acetate in a hydrochloric acid medium has been shown to be the most appropriate extraction method and it has been successfully applied to fish (sea bass) reference material from the National Institute for Environmental Studies (NIES-11) and to a candidate reference material.

Keywords: Marine organisms; Tributyltin; Triphenyltin; Fisetin

1. Introduction

The use of organotin compounds (OTs), such as poly(vinyl chloride) (PVC) stabilisers, fungicides, pesticides, antifouling paint biocides, etc. has led to their entry into the environment. Tributyltin (TBT) and triphenyltin (TPhT), the active agents of OT-based antifouling paints, are the most important OTs in the marine environment. They are toxic to non-target organisms and their effects at very low concentrations on marine biota, such as shell thickening in oysters or imposex on gastropods, have been widely investigated [1]. Furthermore, the continuing contamination of seafood in closed marine areas should also be regarded as a route by which TBT and TPhT may enter the human diet [1]. The recognition of their toxicity has led to regulation of their use in many countries, and to the control of OT levels in environmental samples. Consequently, their analysis is of particular interest and rugged analytical methodologies are required in order to perform effective monitoring programs.

The determination of OTs usually requires the combination of a chromatographic separation technique and a selective and sensitive detection method [2–4]. Gas chromatography is the most used separation technique since its high resolution allows the simultaneous determination of several groups of OTs. Its major drawback is that previous derivatization of polar OTs is mandatory in order to obtain volatile compounds. This step is usually cumbersome and performed off-line. In this sense, ethylation with sodium tetraethylborate has led to important improvements [5–8]. Liquid chromatography (LC) is an alternative that allows the separation of ionic OTs without any previous derivatization step [9]. However, most LC detection systems have a limited sensitivity. At present, inductively coupled plasma–

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mass spectrometry (ICP–MS) detection allows one to achieve enough sensitivity and selectivity for OTs [10–15]. In the case of triorganotin species, fluorimetric detection has been proposed as a cheaper alternative. The use of flavone derivatives as fluorogenic reagents, in combination with micellar media, has been the most successful approach [11,16–19]. Among these fluorimetric methods, post-column derivatization with fisetin has been shown to have excellent sensitivity for TPhT and to be quite satisfactory for TBT, with a high selectivity [18]. However, these fluorimetric methods have rarely been applied to complex matrices such as biological materials.

The extraction of OTs from biological matrices has been achieved using different approaches [4], such as extraction with non-miscible water solvents, which is the most common approach, extraction with polar solvents, basic hydrolysis and enzymatic hydrolysis. When non-polar solvents are used, the addition of an acid and/or a complexing agent is usually required to obtain high recovery values. The most widely used acids are hydrochloric acid and acetic acid. However, some authors suggest that high acid concentrations can lead to degradation of OT [8,20,21], this effect being more important in the case of phenyltin species [21]. Tropolone is the complexing agent that is most extensively used for OT extraction, and its presence generally allows the improvement of recoveries for di- and mono-substituted species, however, it seems to have no significant effect on tri-OT species. Recently, the use of microwave energy has been shown to be a good strategy for decreasing the analysis time in basic hydrolysis [22,23]. Supercritical fluid extraction has also been tested for TBT and TPhT, but results have not been satisfactory [24].

Despite the large number of methods proposed, there is a lack of data about their validation. Moreover, the lability of the phenyltin species, as well as the lack of certified reference materials (CRMs), have led to added difficulties.

The aim of this work is to check the validity of the LC–fisetin–fluorimetric detection method for the determination of TBT and TPhT in biological marine materials. In order to extract the compounds, several extraction methods were tested. The accuracy of the method was evaluated by the analysis of fish (sea bass) reference material from the National Institute for Environmental Studies (NIES-11) CRM. Moreover, the method was used in our laboratory in the certification campaign of a mussel tissue candidate to CRM.

2. Experimental

2.1. Reagents

Stock solutions (1500 mg l^{-1} TPhT) of triphenyltin chloride and (1200 mg l^{-1} TBT) tributyltin chloride were prepared by dissolving the compounds (Fluka, >97% purity, Buchs, Switzerland) in methanol (Baker, HPLC, Deventer, The Netherlands) and these were stored in dark glass bottles at 4°C. Standard solutions were diluted 50-fold with methanol on a weekly basis and these were also stored at 4°C. Subsequent dilutions were freshly prepared with methanol. Calibrants for the certification exercise were supplied by the European Commission [25].

The LC mobile phase was prepared by mixing 100 ml of an aqueous solution of 0.75 *M* ammonium acetate (Merck, Darmstadt, Germany) with 400 ml of HPLC-grade methanol (Baker). This solution was filtered through a 0.2-μm Nylon membrane filter (Lida, Kenosha, WI, USA) and degassed for 10 min using a helium current.

The post-column reagent used for LC fluorimetric detection contained $5 \cdot 10^{-5}$ *M* fisetin and $2.4 \cdot 10^{-2}$ *M* Triton X-100. This was prepared from a $2.5 \cdot 10^{-3}$ *M* methanolic solution of fisetin (Aldrich, Steinheim, Germany) and a 0.2 *M* aqueous solution of Triton X-100 (Merck).

Protease Type XIV and lipase Type VII enzymes were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate, hydrochloric acid, sodium chloride, sodium hydrogencarbonate, citric acid monohydrate, ammonium dihydrogenphosphate, ethanol (Merck, p.a.) and methanol (Baker, HPLC-grade) were used in extraction experiments.

Double-deionized water (Milli-Q, Millipore, Molsheim, France), with a resistivity of 18.2 MΩ cm^{-1}, was used throughout.

All glassware used for experiments was soaked

previously in 10% nitric acid for 24 h and rinsed with double-deionized water.

2.2. Apparatus

The LC equipment consisted of a Model 480 double piston pump (Ginkotek, Germering, Germany), a Ginkotek MSV 6 injection valve equipped with a 200-μl loop and a Partisil SCX (10 μm particle size, 25 cm×4.6 mm I.D.) analytical column (Whatman, Maidstone, UK) with a guard column. An Aminco-Bowman Series 2 spectrofluorimeter (SLM Aminco, Rochester, NY, USA), equipped with a 25-μl flow-cell (Hellma, Müllheim, Germany), was used for LC detection.

A rotary mixer 34526 (Brenda Scientific, Breda, Netherlands), a 4239 R high speed refrigerated centrifuge (ALC, Milan, Italy) with ALC 6050 polyethylene tubes, a LaboRota 300 rotavapor (Resona, Germany) with a Labo-therm SW 200 thermostatic bath (Resona) and an ultrasound bath (Selecta, Abrera, Spain) were used in extraction experiments.

2.3. Procedures

2.3.1. Extraction procedures

2.3.1.1. Method A

Method A was adapted from the method proposed by Gómez-Ariza et al. [26]. To 0.2–1 g of sample in a 40-ml polyethylene tube, 20 ml of methanol were added and the mixture was sonicated for 15 min. The methanolic phase was separated by centrifugation (13 000 *g*, 15°C, 15 min) and the supernatant was removed with a pasteur pipette. The residue was extracted again, as previously described, and, finally, it was washed with 5 ml of methanol. The extracts were combined and reduced to about 2 ml by rotary evaporation. Finally, the volume was made up to 5 ml with methanol.

2.3.1.2. Method B

Method B was adapted from the method proposed by Tsuda et al. [27]. To 0.4 g of mussel sample in a 80-ml polyethylene extraction tube, 10 ml of ethyl acetate and 20 ml of an aqueous solution containing 2.6 *M* NaCl and 0.6 *M* HCl were added. The mixture was stirred mechanically for 30 min and then centrifuged (13 000 *g*, 15°C, 15 min). The organic phase was transferred with a pasteur pipette to a 40-ml glass tube with a PTFE liner. The remaining residue was washed with 5 ml of ethyl acetate and the organic layer was added to the previous extract. This extract was washed for 2 min with 10 ml of an aqueous phase containing 0.5 *M* $NaHCO_3$ and 1.3 *M* NaCl. The tube was centrifuged (1200 *g*, 5 min) and the organic phase was transferred to an evaporation flask. The aqueous phase was shaken with 5 ml of ethyl acetate. The organic phases were pooled and evaporated just to dryness in a rotary evaporator at 35°C. The residue was reconstituted with 2–3 ml of methanol (dilution was on a mass basis).

2.3.1.3. Method C

Method C was described by Ceulemans et al. [8]. Briefly, a 0.1-g tissue sample was placed in an 8-ml glass test-tube, together with protease and lipase (100 mg each). Then, 4 ml of citrate–phosphate buffer, pH 7.5, was added and the mixture was stirred magnetically for 4 h at 36–37°C.

2.3.2. LC–fluorimetric determination

A 200-μl volume of reconstituted extract was injected into the LC system and quantitation was performed by means of calibration graphs obtained in the peak area mode from methanolic solutions of TBT and TPhT (25–2000 μg l^{-1} for TBT and 0.3–600 μg l^{-1} for TPhT). The operating conditions used are summarised in Fig. 1.

2.3.3. Spiking procedure

A 2-ml volume of methanol was added to 0.5 g of dry mussel tissue, to obtain a slurry. A 1-ml volume of a methanolic solution containing TBT and TPhT was added dropwise to the slurry. The mixture was left overnight with orbital shaking, preserved from light. The solvent was evaporated by means of a nitrogen stream.

2.4. Samples

The sample used was lyophilized mussel from the gulf of La Spezia (Italy). Fish (sea bass) reference material was from the National Institute for Environmental Studies (NIES-11), with a certified concentration of TBT (1.3±0.1 μg g^{-1} TBTCl) and

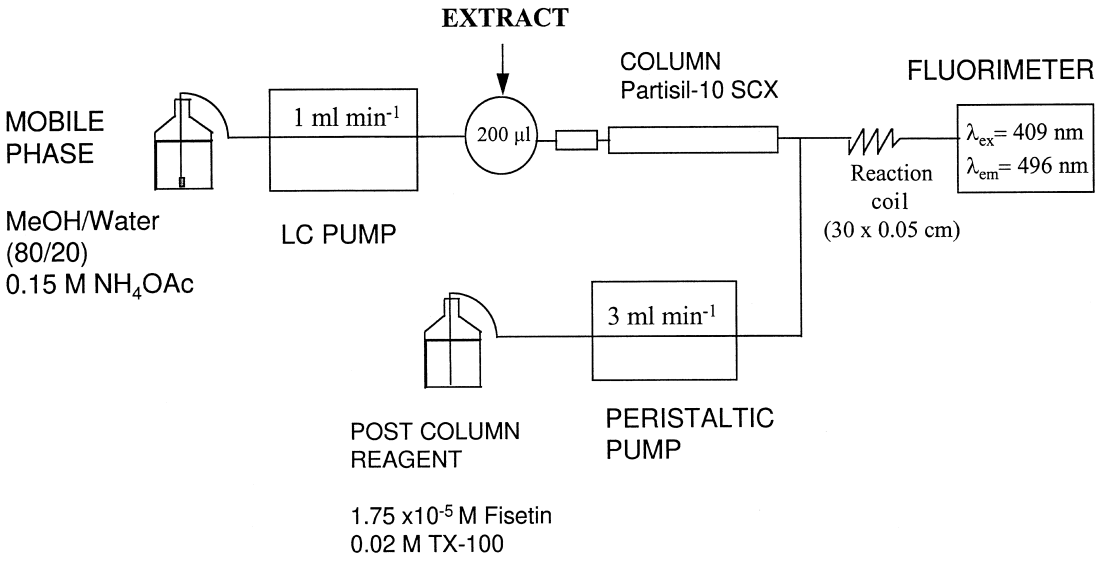


Fig. 1. Scheme of the LC–fluorimetry set-up and operating conditions.

a reference concentration of TPhT (6.3 μg g^{-1} TPhTCl). Lyophilized mussel reference material (*Mytilus edulis*) from the European Commission (CRM-477), with a certified value of TBT (2.20±0.19 μg g^{-1}), was also used. All samples were stored at −20°C.

3. Results and discussion

In order to apply the LC–fluorimetric detection method to the analysis of biological materials, three extraction methodologies, previously described, were tested. Method A is based on the use of methanol and sonication, and it has been reported that it provides good results for TBT extraction from mollusc samples [26]. Method B uses ethyl acetate and hydrochloric acid, i.e. a non-water-miscible solvent plus acid. It has been successfully applied to the analysis of OTs in fish tissues [12,27,28]. Method C is an enzymatic procedure based on the hydrolysis of biological tissues by lipase and protease, and the subsequent release of the OTs into the solution. It has been applied to the analysis of OTs in fish and shellfish samples [8,29,30].

First, extraction methods A and B were applied to a mussel sample with TBT and TPhT and the results obtained are shown in Table 1. On comparing the results, it can be seen that both methods led to similar TBT values, whereas method B provided a higher TPhT concentration than method A.

Next, the procedures were applied to a fish reference material (NIES-11). When method A was applied to this material, neither TBT nor TPhT were detected in the extract. In order to ascertain whether the extraction or the determination step was responsible for the result, the analytes were added to NIES-11 extracts and, in this case, recoveries of about 100% were obtained for both compounds. This indicated that there were matrix problems that hindered the extraction but not the determination. This problem could be attributed to differences in the composition of the matrix between the mussel and

Table 1
Determination of TBT and TPhT in a mussel sample and in NIES-11 by LC–fluorimetry

Method[a]	Sample	TBT (μg g^{-1})	TPhT (μg g^{-1})
A (n=2)	Mussel	1.80±0.07	0.87±0.08
B (n=4)	Mussel	1.60±0.27	1.31±0.01
B (n=3)	NIES-11[b]	1.06±0.04[c]	6.04±0.08[c]

[a]The number of replicates is given in parentheses.
[b] The certified value for TBT is 1.3±0.1 μg g^{-1} (as TBTCl) and the reference value for TPhT is 6.3 μg g^{-1} (as TPhTCl).
[c] Concentrations are expressed in μg g^{-1} as the chloride (TBTCl or TPhTCl).

the fish tissue, mainly in respect of their lipid contents.

The results obtained on analysis of NIES-11 using extraction method B are also given in Table 1. Recoveries of 82% for TBT and of 96% for TPhT, in relation to the certified and the reference values, respectively, were obtained.

In order to reach a concentration level that was suitable for detection, when method B was applied to samples with a low content of TBT and TPhT, the extract was reconstituted in a smaller volume of liquid. In this case, it was observed that the small amount of acid remaining in the extracts caused differences between the retention times of TBT and TPhT in the samples and the standards. In order to prevent this effect, the ethyl acetate extract was washed with an aqueous solution of $NaHCO_3$ and NaCl. The effectiveness of this step was demonstrated because TBT and TPhT peaks from standards and samples eluted at the same retention time (Fig. 2). Moreover, it was observed that the washing step led to a cleaner extract and, thus, a cleaner chromatogram was obtained. In order to determine the influence of this modification on the accuracy of the procedure, the recoveries of known amounts of the analytes added to extracts before and after the washing step were assessed. No significant differences between them were observed.

Finally, the enzymatic hydrolysis method, method C, was applied to NIES-11. Injection of the aqueous solution resulting from the hydrolysis provided a distorted chromatogram, where neither TPhT nor TBT were identified. The addition of both compounds to the extract did not result in significant

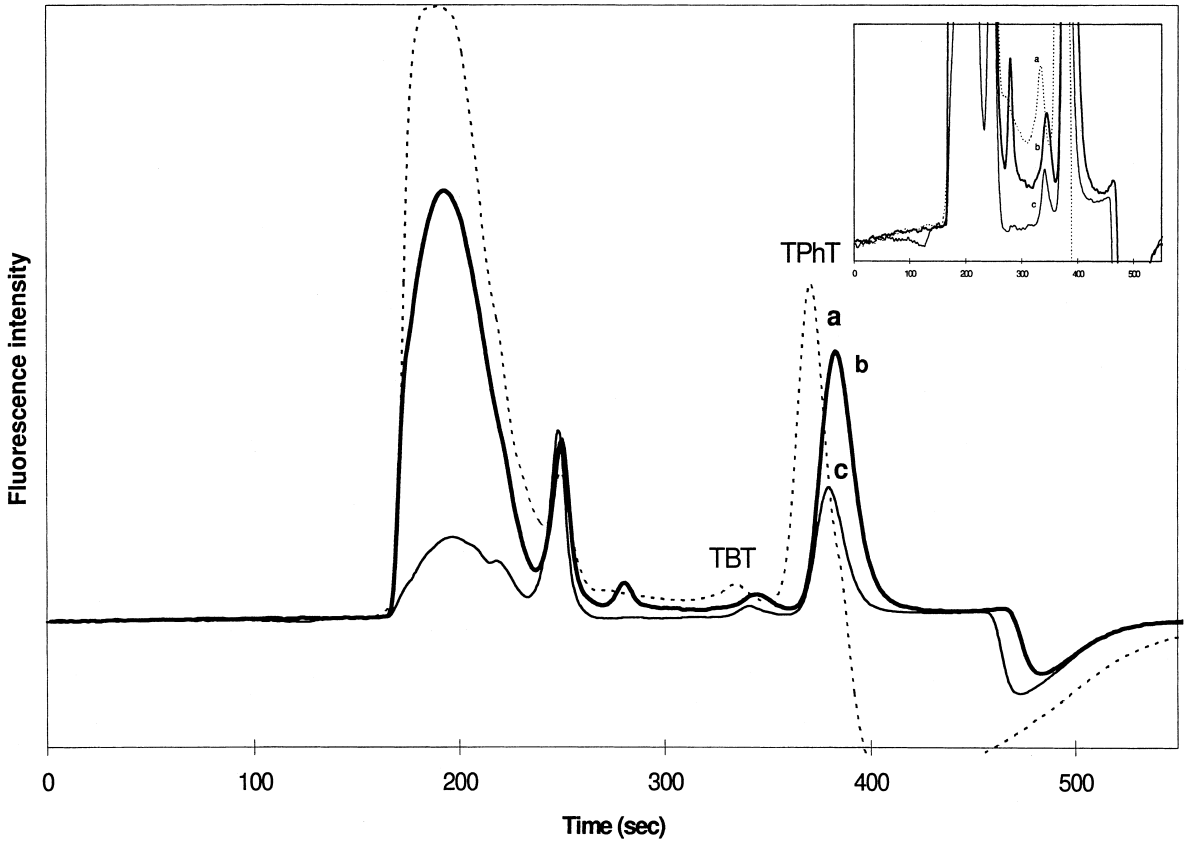


Fig. 2. Chromatograms of (a) an extract of mollusc without washing, (b) an extract of mollusc after washing, (c) a standard solution containing TBT (113 ng g^{-1}) and TPhT (59 ng g^{-1}). A detailed view of the TBT peaks is shown in the window. Experimental conditions are given in the scheme of Fig. 1.

changes in the chromatogram. It seems that the high concentration of citrate in the extract interferes with the fluorimetric detection.

Enzymatic hydrolysis was later used as a pretreatment step in method B. The combined procedures were applied to the analysis of NIES-11, and recoveries were only slightly higher than those obtained with method B. Moreover, chromatograms provided by enzymatic hydrolysis–ethyl acetate extraction were not cleaner than those obtained by direct injection of the extract obtained using the ethyl acetate method. Therefore, the use of enzymatic hydrolysis as a pretreatment step was not considered in further experiments.

This study indicated that, among the assayed extraction methods, method B, which makes use of ethyl acetate, was the one that provided the best results. It is worth noting that the combination of extraction method B and LC–fluorimetry is one of the few reported methods that have been used for TPhT determination in NIES-11. The proposed method led to excellent repeatability for TPhT, with a R.S.D. (%) of less than 2% (Table 2).

3.1. Analysis of a candidate reference material

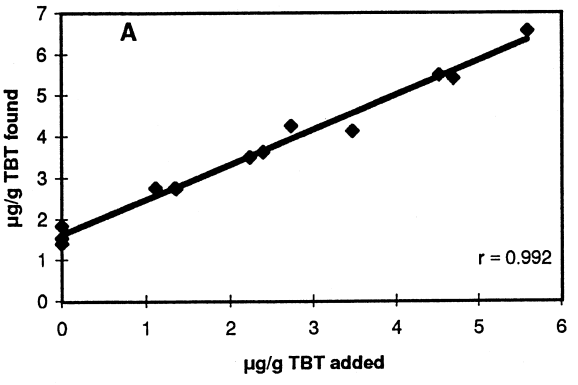
In agreement with previous experiments, method B was chosen for the analysis of a mussel CRM.

In order to evaluate the recovery of the analytes, spiking experiments were carried out according to the procedure described in Section 2. Samples were spiked at three levels with amounts of TBT and TPhT that were approximately once, twice and three times the incurred concentrations. For each experiment, the recoveries were calculated from regression analysis of the data points ($\mu g\ g^{-1}$ OT added vs. $\mu g\ g^{-1}$ OT found), the slope of the regression line being the recovery of the OT species. The whole recovery experiment was repeated three times to evaluate its reproducibility. Fig. 3 shows all of the results obtained, which were put together in one graph. The overall recovery was calculated from the slope of the graph and the reproducibility was determined from the standard deviation of the slope. Good correlation was observed between the added and found concentrations. The recoveries obtained were 81±3% and 87±2% for TBT and TPhT, respectively.

Replicate determinations of the candidate reference material were performed on the same day and also on different days (Table 3). The within-day repeatabilities were 6 and 2% for TBT and TPhT, respectively. The between-day reproducibilities were slightly higher, being 7 and 4% for TBT and TPhT, respectively. These values compare favourably with the mean standard deviation obtained by other laboratories.

Table 2
Survey of reported data on TBT and TPhT concentrations in NIES-11

Extraction method	Determination technique	TBTCl ($\mu g\ g^{-1}$)	TPhTCl ($\mu g\ g^{-1}$)	Reference
HCl–EtOAc	LC–ICPMS	1.2	6.2	[12]
SFE	LC–ICPMS	0.57	1.45	[24]
Enzymatic hydrolysis	Et–GC–AED	1.36±0.07	7.26±0.04	[8]
TMAH	Et–GC–AED	1.34±0.07	5.91±0.44	[8]
0.5 *M* HCl in MeOH (ultrasound)	Et–CT–QFAAS	1.09±0.04		[31]
0.5 *M* HOAc in MeOH (ultrasound)	Et–CT–QFAAS	1.26±0.04		[31]
Enzymatic hydrolysis	HG–GC–AAS	1.13±0.01		[32]
0.1 *M* HCl in MeOH (ultrasound)	HG–GC–AAS	1.05±0.02		[32]
Enzymatic hydrolysis	Et–GC–FPD	1.04±0.04		[32]
TMAH (microwaves)	Et–GC–AED	1.22±0.04		[22]
HOAc (microwaves)	Et–GC–AED	1.37±0.16	5.80±0.81	[33]
HOAc (microwaves)	Et–GC–FPD	1.18±0.24	5.41±0.85	[33]
		1.32±0.05	6.18±0.74	
TMAH (microwaves)	Et–GC–QFAAS	1.20±0.10		[34]
HCl–EtOAc	LC–fluorimetry	1.06±0.04	6.04±0.08	This work



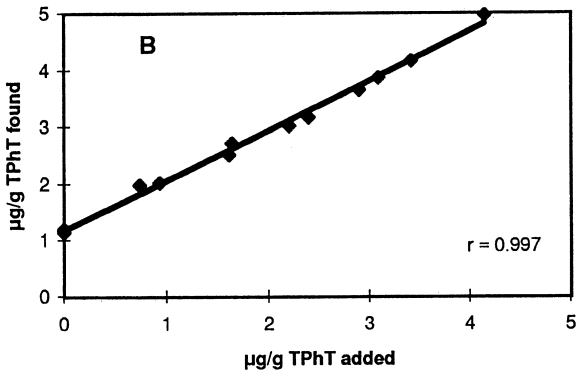


Fig. 3. Recovery experiment graph for TBT (A) and TPhT (B) in the mussel candidate reference material. Correlation factors for the regression lines are indicated in the graphs.

The TBT concentration obtained using ethyl acetate extraction and LC–fluorimetry, after correction for recovery, was in good agreement with the mean value calculated from eighteen laboratories (2.20 ± 0.19 µg g^{-1} TBT) [25]. In the case of TPhT, due to the large spread of results found by the different laboratories and some instability risks, it was decided not to give any value for this analyte [25].

Table 3
Quantitation of TBT and TPhT in the mussel candidate reference sample using ethyl acetate extraction and LC–fluorimetry

	Concentration[a] (µg g^{-1} as cation)	
	TBT	TPhT
Day 1 ($n=4$)	2.17 ± 0.04	1.41 ± 0.04
Day 2 ($n=2$)	2.44 ± 0.04	1.51 ± 0.06
Day 3 ($n=3$)	2.32 ± 0.14	1.49 ± 0.01
Mean value±SD $n=9$	2.27 ± 0.15	1.46 ± 0.06
Certified value	2.20 ± 0.19	–

[a] Concentrations were corrected by recoveries.

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